



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁶ : C12N 15/11, 9/00, A61K 31/70, 48/00	A2	(11) International Publication Number: WO 95/03406 (43) International Publication Date: 2 February 1995 (02.02.95)
(21) International Application Number: PCT/US94/08334 (22) International Filing Date: 19 July 1994 (19.07.94) (30) Priority Data: 08/093,800 19 July 1993 (19.07.93) US (71) Applicant: GEN-PROBE INCORPORATED [US/US]; 9880 Campus Point Drive, San Diego, CA 92121 (US). (72) Inventors: DATTAGUPTA, Nanibhushan; 4221 Kerwood Court, San Diego, CA 92130 (US). SRIDHAR, C., Nagaraja; 1911 East Larch Street, Simi Valley, CA 93065 (US). KACIAN, Daniel, L.; 3911 Tambor Road, San Diego, CA 92124 (US). (74) Agents: HEBER, Sheldon, O. et al.; Lyon & Lyon, First Interstate World Center, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).		(81) Designated States: AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: ENHANCEMENT OF OLIGONUCLEOTIDE INHIBITION OF PROTEIN PRODUCTION, CELL PROLIFERATION, AND/OR MULTIPLICATION OF INFECTIOUS DISEASE PATHOGENS (57) Abstract The invention features the use of a purified first targeted oligonucleotide in combination with either 1) a subtargeted oligonucleotide, 2) second targeted oligonucleotide, or 3) a non-targeted phosphorothioate oligonucleotide, to inhibit protein production of a targeted nucleic acid sequence, cell proliferation, and/or the multiplication of a foreign organism. The subtargeted oligonucleotide contains a truncated version of the targeted oligonucleotide.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

DESCRIPTIONEnhancement Of Oligonucleotide Inhibition Of
Protein Production, Cell Proliferation, And/Or
Multiplication Of Infectious Disease Pathogens5 BACKGROUND OF THE INVENTION

This invention relates to enhancing the ability of oligonucleotides, such as antisense oligonucleotides and ribozymes, to inhibit protein production, cell proliferation, and/or the multiplication of a foreign organism.

10 Oligonucleotides such as antisense oligonucleotides and ribozymes can hybridize to a target RNA, such as mRNA, and inhibit production of protein from that RNA. Numerous mechanisms have been proposed to explain the effects of antisense oligonucleotides. For example, see
15 Helene, C. and Toulme, J. Biochimica et Biophysica Acta 1049:99 (1990), and Uhlmann, E. and Peyman, A. Chemical Reviews 90:543 (1990). Proposed mechanisms include formation of a DNA:RNA substrate for cellular RNase H, which degrades the RNA strand involved in the duplex;
20 hybridization of an antisense oligonucleotide to nascent mRNA leading to premature transcription termination; and interfering with mRNA processing by hybridizing to a pre-mRNA intron/exon junction. These and several other proposed mechanisms for inhibiting nucleic acid activity by
25 antisense oligonucleotides are based upon the ability of antisense oligonucleotides to hybridize to a target RNA sequence.

Oligonucleotides can also inhibit viruses by a sequence non-specific mechanism. For example, Majumdar,
30 et al., Biochemistry 28:1340 (1989) describe the use of phosphorothioate oligonucleotides to inhibit HIV reverse transcriptase.

Hybridization of an oligonucleotide to a target nucleic acid sequence occurs by hydrogen bonding between
35 complementary nucleotides present on the oligonucleotide and the nucleic acid target site. If the individual nucleotides on the target nucleic acid sequence are not

accessible to the oligonucleotide, hybridization will be prevented. Inaccessibility of nucleotides can be due to various factors such as secondary structure of the target nucleic acid and proteins associated with target nucleic acid.

Hogan, et al., U.S. Patent No. 5,030,557, describe the use of a "helper" oligonucleotide to help a probe oligonucleotide hybridize to its target nucleic acid sequence in a diagnostic assay.

Goodchild, J., Abstract in Third International Symposium On Catalytic RNAs (Ribozymes) And Targeted Gene Therapy For The Treatment Of HIV Infection, San Diego CA (1992), describes the use of "facilitators" to improve the performance of hammerhead ribozymes. Facilitators are oligoribonucleotides that bind to a RNA substrate target sequence contiguously with a ribozyme binding site.

Tullis, U.S. Patent No. 5,023,243, provides a general description of use of antisense oligonucleotides. Kaji, U.S. Patent No. 4,689,320, provides data allegedly showing a decrease in mortality in mice infected with herpes simplex virus when treated with an antisense oligonucleotide having a nucleotide sequence targeted to herpes simplex virus. Goodchild et al., U.S. Patent No. 4,806,463, provide data to demonstrate the ability of several different specified antisense oligonucleotides to inhibit HTLV-III (HIV) replication and gene expression in cultured cells infected with HIV. Cantin et al., U.S. Patent. No. 5,110,802, describe the use of a particular methylphosphonate-linked oligonucleotide to inhibit HIV replication (these U.S. patents are hereby incorporated by reference herein).

Matsukura et al., Proc. Natl. Acad. Sci. 86:4244, describe inhibition of HIV expression without killing the host cell using a phosphorothioate-linked oligonucleotide targeted to a rev sequence. Matsukura et al. also discuss using oligonucleotides joined by linkages other than phosphodiester or phosphorothioate groups. According to

Matsukura et al., "certain chemically modified oligomers (e.g., methylphosphonates) have inherent limitations in that they are quite insoluble and require exceedingly high concentrations for biological effects."

5 Ribozymes, like antisense oligonucleotides, can be targeted to specific nucleic acid sequences based upon hybridization to a complementary nucleic acid sequence target site. The hybridized ribozyme can inhibit protein production from a target nucleic acid sequence by cleaving
10 the nucleic acid sequence. Rossi et al., Aids Research And Human Retroviruses 8:183 (1992), in discussing using ribozymes as possible anti-HIV therapeutic agents, mention potential factors which may decrease accessibility of a target site. These factors may include HIV RNA having
15 extensive secondary structure and cellular proteins associated with RNA.

 Ribozymes and antisense oligonucleotides can be used to inhibit the functioning of nucleic acid sequences present in different organisms such as bacteria, plants,
20 fungi, animals, or humans. For example, Fung et al., entitled "Gene therapy for cell proliferative diseases," PCT/US91/02478, discuss using ribozymes in humans to inhibit the functional expression of positive-acting growth regulatory elements to treat pathological cell
25 proliferative diseases. Fung et al. also mention using antisense RNA as a means of suppressing gene expression.

SUMMARY OF THE INVENTION

 The present invention features methods and compositions for enhancing the ability of an oligonucleo-
30 tide to inhibit protein production, cell proliferation and/or the multiplication of a foreign organism. The featured oligonucleotide compositions have a first targeted oligonucleotide and either, 1) a subtargeted oligonucleotide, 2) a second separately targeted
35 oligonucleotide, or 3) a non-targeted phosphorothioate oligonucleotide.

The first targeted oligonucleotide is designed to hybridize to a target nucleic acid sequence. Hybridization between an oligonucleotide and a nucleic acid sequence occurs by complementary hydrogen bonding.

5 While targeted oligonucleotides are designed to hybridize to a nucleic acid sequence, the effect of a targeted oligonucleotide on protein synthesis and/or viral multiplication is not limited to effects caused by hydrogen bonding to a complementary nucleic acid sequence.
10 For example, targeted oligonucleotides may inhibit protein catalytic ability.

The subtargeted oligonucleotide is designed to hybridize to a subtarget region of the target region of the first targeted oligonucleotide. Thus, a subtargeted
15 oligonucleotide contains a truncated portion of a first targeted oligonucleotide. The ability of a subtargeted oligonucleotide, like a targeted oligonucleotide, to inhibit protein synthesis and/or viral multiplication is not limited to a mechanism involving hydrogen bonding
20 between nucleotides.

The second targeted oligonucleotide is designed to hybridize to a different region than the first targeted oligonucleotide. Preferably, the target region of the second targeted oligonucleotide is located more than three
25 nucleotides away from the target region of the first targeted oligonucleotide.

Targeted oligonucleotides, such as antisense oligonucleotides and ribozymes, can be designed to hybridize to cellular and viral nucleic acid sequences
30 based upon complementary hydrogen bonding between nucleotides. The hybridized oligonucleotide then inhibits the nucleic acid activity of the target nucleic acid.

"Non-targeted phosphorothioate oligonucleotide" refers to an oligonucleotide joined by phosphorothioate
35 linkages, which can function in a non-sequence specific manner. For example, see Majumdar, et al., supra, hereby incorporated by reference herein.

The described oligonucleotide compositions are particularly useful for inhibiting the nucleic acid activity of a target nucleic acid. The activity of target nucleic acid is that activity needed for protein
5 production or nucleic acid replication. Target nucleic acid includes nucleic acid present in different types of organisms such as bacteria, plants, fungi, parasites, viruses, and animals. Generally, the activity of target nucleic acid includes transcription of RNA, translation of
10 mRNA, processing of mRNA, stability of mRNA, and replication of both RNA and DNA.

The activity of viral nucleic acid is that activity needed for viral multiplication. For example, HIV nucleic acid activity needed for replication includes
15 reverse transcription of HIV RNA, translation of HIV mRNA, processing of HIV mRNA, packaging of genomic HIV RNA, and HIV RNA stability. The importance of a particular type of nucleic acid activity can vary in different types of viruses.

20 Thus, in a first aspect, the invention features a method and compositions for reducing the level of, or inhibiting, protein production coded for by a target nucleic acid. The method involves using a purified targeted oligonucleotide and a purified subtargeted
25 oligonucleotide. These oligonucleotides are targeted to a nucleic acid sequence present on the target nucleic acid. Target nucleic acid sequences include mRNA coding for a protein or corresponding genomic and/or regulatory sequences. The subtargeted oligonucleotide comprises a
30 truncated version of the targeted oligonucleotide.

By "purified" is meant to exclude naturally occurring oligonucleotides. Purified oligonucleotides may be produced by techniques known in the art such as chemical synthesis and by *in vitro* or *in vivo* expression from
35 recombinant nucleic acid molecules, e.g., retroviral vectors.

Subtargeted oligonucleotides are targeted to a subset of the targeted oligonucleotide target region. Figure 1 illustrates a target region and gives some examples of subset target regions. The double line in Figure 1 represents a target region or a subset target region. The subset target region is that part of the subtargeted oligonucleotide target region which is also present in the target region of the targeted oligonucleotide. The subtargeted oligonucleotide may also have nucleotides which are not present in the targeted oligonucleotide target region. The solid line in Figure 1 represents additional nucleotides which can make up a target site for a subtargeted oligonucleotide.

By "comprises a truncated version" is meant that the subtargeted oligonucleotide is complementary to only a subset target region and may contain additional nucleotides not complementary to the target region.

Figure 2 provides an example of a target region, a subtargeted oligonucleotide, and a targeted oligonucleotide. The subtargeted oligonucleotide contains part of the nucleic acid sequence of the targeted oligonucleotide. The region of complementarity between the subtargeted oligonucleotide and the target region is the subset target region.

Without being bound to any theory, subtargeted oligonucleotides are thought to enhance inhibition of protein synthesis and/or viral multiplication primarily by hybridizing to nucleic acid sequences or binding to protein regions, inaccessible to the corresponding targeted oligonucleotide. For example, the formation of a subtargeted oligonucleotide:target duplex may increase the chance of forming a targeted oligonucleotide:target duplex, and/or inhibit a different form of target nucleic acid than the targeted oligonucleotide. Different forms of RNA may arise due to nucleic acid processing (e.g., splicing of mRNA), and changes in the intracellular environment. Changes in the intracellular environment

include different microenvironments within the cell such as in the cytoplasm versus the nucleus, and the association of proteins with nucleic acid at different stages of RNA processing.

5 Preferably, the subtargeted oligonucleotide, as illustrated in Figure 2, contains only nucleotides present in the targeted oligonucleotide and is targeted to a subset target region 40 to 60 percent the size of the target region. Presumably, as the subtargeted
10 oligonucleotide becomes larger, it is less likely to hybridize to regions inaccessible to targeted oligonucleotides.

The subtargeted oligonucleotide should be 7 nucleotides or more in length. The smaller the
15 subtargeted oligonucleotide, the more likely the subtargeted oligonucleotide will hybridize to non-target sequences. Hybridization to non-target sequences may lead to unwanted toxicity and thus should be avoided.

The sugar groups of the nucleotide subunits of
20 the oligonucleotides described herein may be ribose, deoxyribose, or modified derivatives thereof such as O-methyl ribose. The nucleotide subunits may be joined by linkages such as phosphodiester linkages, modified linkages, or by non-nucleotide moieties that do not
25 prevent hybridization of the oligonucleotide. Modified linkages include those linkages in which a standard phosphodiester linkage is replaced with a different linkage, such as a phosphorothioate linkage, a methylphosphonate linkage, a phosphodithioate linkage, or
30 a phosphoselenate linkage.

The oligonucleotide combinations described herein can be made up of two oligonucleotides each having a different predominant linkage. "Predominant linkage" refer to the type of linkage present in the highest
35 percentage, of the total linkages, in an oligonucleotide. Preferably, the oligonucleotide contains linkages resistant to exonuclease degradation such as

methyolphosphonate or phosphorothioate linkages. More preferably, the predominant linkage of the oligonucleotide is resistant to exonuclease degradation.

In another aspect, a method and compositions for inhibiting protein production is provided using two purified targeted oligonucleotides. The two purified oligonucleotides are targeted to different nucleic acid sequences coding for the same protein. Preferably, the two oligonucleotides are targeted to nucleic acid sequences more than 3 nucleotides apart.

In another aspect, a method and compositions for inhibiting the multiplication of a foreign organism is provided. The described methods include the use of the featured compositions described above; 1) two purified targeted oligonucleotides, 2) a purified targeted oligonucleotide and a purified subtargeted oligonucleotide, and 3) a purified targeted oligonucleotide and a non-targeted phosphorothioate oligonucleotide. The two purified oligonucleotides are targeted to different nucleic acid sequences. Preferably, these different nucleic acid sequences are more than 3 nucleotides apart.

Foreign organisms are those organisms, such as bacteria, fungi, parasites, and viruses, which may infect higher level organisms such as humans, animals, and plants. Parasites include organisms living in or on another organism such as a plant, animal, or human. Animal and human parasites of medical importance include intestinal protozoa such as amebas, flagellates, coccidia, and microsporidia; blood protozoa such as malaria; and helminths such as nematodes, cestodes, and trematodes.

In another aspect, a method and compositions for treating cell proliferative diseases is described. Cell proliferative diseases include inappropriate pathological cell growth such as exists in cancer cells, malignant cell proliferation and benign proliferative diseases. The use of oligonucleotides to treat cell proliferative diseases

is described by Fung et al., supra, hereby incorporated by reference herein. The methods and compositions described herein enhance the ability of oligonucleotides to inhibit synthesis of a regulatory element responsible for cell
5 proliferative disease. By "regulatory element" is meant a component of the cell encoded for by nucleic acid that controls cell growth and/or cell division.

In another aspect, a therapeutic composition comprising a purified targeted oligonucleotide consisting
10 essentially of from 9 to 100 nucleotides able to hybridize with the HIV nucleic acid SEQ. ID. NO. 5: CCCGAGGGGA CCCGACAGGC CCGAAG and a purified subtargeted oligonucleotide is provided. The subtargeted oligonucleotide includes a truncated version of the
15 targeted oligonucleotide. The therapeutic composition is able to reduce the normal activity of HIV nucleic acid needed for viral multiplication.

By "hybridize" is meant that the oligonucleotide can form a duplex with its target nucleic acid to reduce
20 the normal activity of the target nucleic acid. Hybridization between anti-viral oligonucleotide and target HIV nucleic acid is preferably specific to target viral nucleic acid under *in vivo* conditions. However, anti-viral oligonucleotides that can form a duplex with
25 both the viral target site and non-target cellular sites under *in vivo* conditions may still be useful as anti-viral agents *in vivo*. *In vivo* effectiveness may arise due to different factors such as adequate discrimination between inhibition of viral nucleic acid activity and cellular
30 nucleic acid activity.

By "consisting essentially of" is meant to include oligonucleotides having sufficient contiguous nucleotides complementary to the target sequence to hybridize under physiological conditions and any
35 additional nucleotides associated therewith which do not prevent (and preferably do not enhance) such hybridization. Preferably, consisting essentially of a

nucleic acid sequence means the oligonucleotide contains a nucleic acid sequence which has 0 to 5% nucleotide base difference (excluding RNA or DNA derivatives), from a specified sequence, has the claimed activity (e.g., anti-
5 HIV activity), and may contain up to 4 additional nucleotides. The additional nucleotides may be complementary or non-complementary to HIV nucleic acid.

"RNA and DNA derivatives" refer to RNA and DNA molecules having the same complementary base pair
10 hybridization properties. RNA and DNA derivatives have different sugar groups (i.e., ribose versus deoxyribose), and may differ by the presence of uracil in RNA and thymine in DNA.

The targeted oligonucleotide preferably has the
15 nucleic acid sequence given by SEQ. ID. NO. 1 or the RNA derivative thereof SEQ. ID. NO. 2. The subtargeted oligonucleotide is preferably 40-60% the size of SEQ. ID. NO. 1. More preferably, the subtargeted oligonucleotide is given by SEQ. ID. NO. 3 or the RNA derivative thereof
20 SEQ. ID. NO. 4. The invention also features methods for use of the above oligonucleotides and vectors encoding such oligonucleotides.

A specific application of these methods and compositions is described for the inhibition of HIV
25 nucleic acid activity. The use of a subtargeted oligonucleotide synergistically increased the effect of an antisense oligonucleotide, thereby increasing the efficacy of the antisense oligonucleotide to inhibit HIV multiplication. Advantages of increased efficacy include
30 reducing the effective concentration of antisense oligonucleotide needed for clinical treatment.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

35 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates several examples of subset target regions. The double line represents nucleic acid

in the target region. The single line represents nucleic acid outside the target region (part of the subtargeted oligonucleotide target region).

Figure 2 illustrates target site 1, a subtargeted oligonucleotide and a targeted oligonucleotide.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention describe methods and compositions for synergistically inhibiting protein production and/or the multiplication of a foreign organism. The featured oligonucleotide compositions include the combination of a first targeted oligonucleotide and either 1) a subtargeted oligonucleotide, 2) a second targeted oligonucleotide, or 3) a non-targeted phosphorothioate oligonucleotide.

The featured oligonucleotide compositions may also be used in conjunction with additional targeted and/or subtargeted oligonucleotide(s). Additional targeted oligonucleotide(s) are targeted to a different nucleic acid sequence than the other targeted oligonucleotide(s) present in the initial composition. Similarly, additional subtargeted oligonucleotide(s) are targeted to a different subtarget region than the other subtarget oligonucleotide(s) present in the initial composition. For example, a second targeted or subtargeted oligonucleotide may be used in conjunction with a targeted oligonucleotide/subtargeted oligonucleotide composition. Similarly, a third targeted oligonucleotide can be used in conjunction with a composition containing two targeted oligonucleotides.

Synergistic inhibition refers to more inhibition than the sum of the inhibitions seen with each oligonucleotide alone, when present at the same concentrations as in combination. The present invention describes how to make and use oligonucleotide compositions to synergistically inhibit protein synthesis and/or viral multiplication.

Preferably, the synergistic inhibition is at least 10 fold greater than the sum of the individual oligonucleotide inhibitions. More preferably the synergistic inhibition is at least 100 fold. Most preferably the synergistic inhibition is at least 1000 fold.

Numerous uses of targeted oligonucleotides are known, including applications as a human therapeutic, and selection of desirable plant traits. The compositions and methods described herein can be used to enhance the effect of the targeted oligonucleotide in these, and other types, of applications wherein a targeted oligonucleotide is used to inhibit protein production and/or viral multiplication.

An example of using the methods and compositions described herein to inhibit HIV replication is provided below. The example is not limiting in the invention. Those skilled in the art will recognize that equivalent oligonucleotide compositions targeted to different regions in different organisms can be readily designed and synthesized, using this application as a guide.

Proposed Mechanisms

There are numerous mechanisms which can account for the observed synergistic effect. These mechanism include the following:

1. Synergy between the action of longer oligonucleotides working as non-sequence-specific inhibitors (non-targeted phosphorothioate oligonucleotides) and targeted oligonucleotides. Longer length non-targeted phosphorothioate oligonucleotides, for example phosphorothioate C₂₈, as described by Matsukura et al., supra, can inhibit HIV reverse transcriptase in a non-sequence-specific manner. Preferably, the non-target phosphorothioate is 15 nucleotides or longer in length. A mixture of a longer non-targeted phosphorothioate oligonucleotide and a targeted oligonucleotide may show synergy by a combination of non-sequence-specific inhibition of the HIV reverse transcriptase and sequence-

specific (antisense) inhibition of the targeted gene (for example rev or tat). In this instance, the two oligonucleotides would act at different steps in the viral life cycle and on different components of the viral replication/expression machinery.

2. The combination of two oligonucleotides may act in concert to promote uptake of the therapeutic oligonucleotides into the cells. Increased uptake may result from the two types of oligonucleotides combining to form an aggregate, network, or other complex, that is more readily taken up into cells than one type of oligonucleotide alone. These complexes may be non-specific in nature or result from base pairing and stacking of partially complementary regions. Such complexes might bind more readily to receptors. For example, large phosphorothioate oligonucleotides can bind to CD4 receptors and inhibit binding of HIV to the receptor. If binding is mediated through cooperative charge-charge interactions, larger oligonucleotides (and likely complexes of large and small oligonucleotides) should bind more tightly to the cell surface, promoting oligonucleotide uptake.

3. The synergistic effect may be due to one oligonucleotide enhancing the ability of the other to bind in regions of tight secondary structure. The subtargeted oligonucleotide may bind to an open region, not accessible to a targeted oligonucleotide, and then open up a structured region.

For example, the subtargeted oligonucleotide may open up core viral nucleic acid. Retroviruses contain two copies of genomic RNA in each virus particle. These copies are tightly interwound or complexed in the virus core. There may be interstrand base pairing between the two strands. One of the oligonucleotides could bind to one strand and disrupt this interstrand base pairing and thus allow the other oligonucleotide to bind to its target site. This mechanism is based upon both HIV strands

having the same polarity (plus strands), while both the targeted oligonucleotide and the subtargeted oligonucleotide have the same polarity (in this mechanism, minus strands) and are overlapping (one being a truncated
5 version of the other).

4. The smaller subtargeted oligonucleotide might be able to penetrate virus particles; whereas, the larger targeted oligonucleotide may not. Thus, synergy may be due to an effect of the larger oligonucleotide
10 within the infected cell and an effect of the smaller oligonucleotide on events occurring within the highly protected environment of the virus particle.

5. The combination of the two oligonucleotides may promote retention of the two oligonucleotides within
15 the cells. One problem with therapeutic oligonucleotides is back-diffusion or other egress from cells of oligonucleotides. The combination of two oligonucleotides might not interact outside the cell. However, in the changed environment within the cell they might complex or
20 aggregate in such a way as to limit efflux from the cell. Thus, the intracellular concentration of the oligonucleotides may reach and maintain higher levels for a longer period of time with consequent enhancement of their therapeutic activity.

25 6. The two oligonucleotides may act by binding differentially to different proteins which bind mRNA. The subtargeted oligonucleotide might bind better to a portion of the binding protein which is inaccessible to a targeted oligonucleotide, while a longer targeted oligonucleotide
30 might bind better to a more accessible site. For example, a smaller oligonucleotide might bind into an enzyme active site where the larger targeted oligonucleotide would not fit.

On the other hand, a longer targeted
35 oligonucleotide may bind better than a shorter subtargeted oligonucleotide to proteins where accessibility is not a problem. In this instance the increased number of bases

in the longer oligonucleotide may result in increased binding to protein.

Thus, two oligonucleotides, one of which is a truncated version of the other, may synergistically function by each binding more tightly and perhaps exclusively to different proteins. The same mechanism can be applied to binding to nucleic acids where secondary and tertiary structures of the nucleic acids would cause one or the other targeted oligonucleotide molecule to bind more tightly.

7. The molecules might work at different times in the viral life cycle. Different proteins and nucleic acids are expressed, processed, exposed, or otherwise accessible or inaccessible, or important or not important, at various stages of the virus life cycle. Inhibition at more than one stage of the virus life cycle can result in synergy between a subtargeted/targeted oligonucleotide combination, or a two targeted oligonucleotide combination.

8. One oligonucleotide might have faster uptake kinetics than the other, but both might be degraded at the same rate once within the cell. The two oligonucleotides together may maintain an effective intracellular concentration for a longer period of time. The effect would be similar to dosing the patient at intervals. Similar effects should occur if the degradation kinetics or efflux kinetics of the two therapeutic oligonucleotides are different.

9. Oligonucleotides may be able to enter cells through bulk fluid uptake via pinocytosis. Two oligonucleotides could act synergistically (for example by complex formation, differential kinetics, or a non-sequence-specific effect of the larger oligonucleotide on the appropriate transport machinery) to promote efflux of the oligonucleotides from the pinocytotic vesicles into the cell cytoplasm.

10. The two oligonucleotides may bind to different regions within an mRNA molecule coding for a single polypeptide chain or to two different forms of the mRNA molecule. The target sequence may be contained within, for example, a viral genomic RNA molecule and within shorter mRNAs generated by transcription from integrated viral DNA and/or RNA processing. Different structural forms of these RNAs may exist due to intramolecular folding, packaging within virus structures (including those in the process of assembly), and/or association with proteins that bind the nucleic acid. With multiple targeted oligonucleotides, the chance increases that at least one oligonucleotide will bind to the target molecule and affect its activity.

This list of possible mechanisms is not intended to be an exhaustive list. Additional mechanisms are described below to further illustrate the invention.

Subtargeted Oligonucleotide Combinations

A composition made up of a targeted oligonucleotide and a subtargeted oligonucleotide may be used to inhibit production of a particular protein and/or the multiplication of a foreign organism. Subtargeted oligonucleotides are oligonucleotides targeted to a subset of the targeted oligonucleotide target region. These "subset target sites" are smaller versions of the targeted oligonucleotide target region. The subtargeted oligonucleotide may contain additional nucleotides not present in the targeted oligonucleotide which do not prevent hybridization with the subset target region.

Foreign organisms are those organisms, such as viruses, bacteria, fungi, and parasites which may infect higher level organisms such as humans, other animals, and plants. When the foreign organism is a virus, inhibition of viral nucleic acid activity may include inhibiting packaging of nucleic acid, not just inhibition of protein synthesis.

As discussed above, there are several possible mechanisms through which the subtargeted oligonucleotide

may be exerting its effect. Without being bound to any theory, subtargeted oligonucleotides are thought to function primarily by hybridizing to nucleic acid sequences inaccessible to the targeted oligonucleotide.

5 The subtargeted oligonucleotide:target duplex presumably increases the formation of a targeted oligonucleotide:target duplex, and/or inhibits a different form of target nucleic acid than the targeted oligonucleotide.

10 As noted above, different forms of RNA may arise as the RNA moves from one subcellular compartment to another due changes in local microenvironments such as in the cytoplasm versus the nucleus, and the association of proteins at different stages of RNA processing. A
15 subtargeted oligonucleotide may bind to one form of RNA, while a targeted oligonucleotide binds to a different form of RNA.

 In addition, the subtargeted oligonucleotide:subset target duplex may increase the
20 formation of a targeted oligonucleotide:target duplex by opening up the target region to the targeted oligonucleotide, or displacing proteins associated with nucleic acids. Subtargeted oligonucleotides are targeted to a subset of the target sequence. Thus, the subtargeted
25 oligonucleotide may be able to hybridize to a nucleic acid region not accessible to the targeted oligonucleotide.

 However, the targeted oligonucleotide has a longer stretch of contiguous complementary oligonucleotides to the target sequence than the
30 subtargeted oligonucleotide. As a result, the targeted oligonucleotide may be able to displace its corresponding subtargeted oligonucleotide.

 Once formed, the targeted oligonucleotide:target duplex, due to the increased complementarity to the target
35 nucleic acid sequence, may be more stable than a subtargeted oligonucleotide:subset target duplex. A more stable oligonucleotide:target duplex is less likely to be

"knocked" off due to factors such as intramolecular hydrogen bonding or nucleic acid-associated proteins.

Advantages of increased stability of oligonucleotide:RNA duplexes may include increased efficiency of hybrid arrest translation and, when the antisense oligonucleotide is DNA, increased likelihood of recognition and degradation by RNase H. Advantages of increased stability of ribozyme:target duplexes may include keeping the substrate RNA properly aligned for enzymatic digestion.

A synergistic effect may result from the decrease in viable mRNA transcripts by one or more of the mechanisms proposed above. Viable mRNA transcripts refer to mRNA transcripts able to be translated into a complete, functional protein. Oligonucleotides that inhibit the nucleic acid activity of nucleic acid may decrease the number, or ability, of viable mRNA transcripts to code for protein. For example, antisense oligonucleotide hybridization to a pre-mRNA intron/exon junction may prevent processing of pre-mRNA into mRNA, thereby reducing the number of viable mRNA transcripts.

The target region is preferably between 16 and 100 nucleotides sequences in length, more preferably 24 to 50 nucleotides sequences in length. Preferably, the subtargeted oligonucleotide contains nucleotides present in only the targeted oligonucleotide and is targeted to a subset target region 40 to 60 percent the size of the target region. Presumably, as the subtargeted oligonucleotide becomes larger it would act more like the targeted oligonucleotide thereby reducing the synergistic effect of using the targeted/subtargeted oligonucleotide combination. For example, as the subtargeted oligonucleotide increases in size it is less likely to hybridize to a region inaccessible to a targeted oligonucleotide.

The subtargeted oligonucleotide should be 7 or more nucleotides in length. The smaller the subtargeted

oligonucleotide the increased likelihood the subtargeted oligonucleotide will hybridize to non-target sequences. Hybridization to non-target sequences may lead to cell toxicity.

5 The target region for the targeted oligonucleotide preferably forms a secondary structure *in vivo*. Secondary structure has been associated with nucleic acid activity, and thus such regions may be good target sites for inhibiting nucleic acid activity. For
10 example, structured regions of nucleic acids are often sites for recognition by enzymes and binding proteins involved in regulation of gene expression. Regions containing a secondary structure are expected to be less accessible to targeted oligonucleotides. Since
15 subtargeted oligonucleotides may work by hybridizing to targeted regions not accessible to a targeted oligonucleotide, a subtargeted oligonucleotide may have a greater effect on a region involved in a secondary structure than a region not involved in a strong secondary
20 structure. Regions expected to have a secondary structure can be identified using techniques known in the art. (e.g., Zuker, M, in: Methods In Enzymology, vol 190, p. 262 (1979)).

Two Targeted Oligonucleotides

25 A composition comprising two targeted oligonucleotides may be used to inhibit production of a particular protein and/or the multiplication of a foreign organism. The two oligonucleotides are preferably targeted to nucleic acid sequences more than 3 nucleotides
30 apart which may code for the same protein or different proteins. An example of a two targeted oligonucleotide combination which appears to act synergistically, at some oligonucleotide concentrations, is the phosphorothioated oligonucleotide of sequence SEQ. ID. NO:1 and the
35 phosphorothioated oligonucleotide of sequence SEQ. ID. NO:6 (targeted to *pol*).

A targeted oligonucleotide can hybridize to a target nucleic acid sequence region and inhibit production of protein encoded for by the target nucleic acid. While nucleic acids that are not perfectly complementary may hybridize to each other under physiological conditions, the longest stretch of perfect complementarity base sequence generally determines hybrid stability. Oligonucleotides designed to hybridize to a particular sequence should have an appropriate melting temperature (T_m) (the temperature at which 50% of the oligonucleotide is hybridized to its target nucleic acid). The appropriate T_m can be obtained by varying the probe length and nucleotide composition (percentage of G + C versus A + T). The probe length and nucleotide composition should result in a T_m about 2-10°C higher than physiological temperature (37°C).

The longer the complementary region on an oligonucleotide, the more hydrogen bonding to a target sequence, and in general, the higher the T_m . Increasing the percentage of G and C also increases the T_m because G-C base pairs exhibit additional hydrogen bonding and therefore greater thermal stability than A-T base pairs. T_m can be determined using techniques known in the art such as measuring hybridization by the hybridization protection assay (HPA) according to Arnold et al., entitled "Homogeneous Protection Assay," EPO application number 88308767.8, publication number 309230, and Nelson et al., in Nonisotopic DNA Probe Techniques, p. 275 Academic Press, San Diego (Kricka, ed., 1992) (these references are hereby incorporated by reference herein). Oligonucleotides can be labeled with acridinium ester derivatives as described by Arnold, et al., PCT/US88/03361, entitled "Acridinium Ester Labeling and Purification of Nucleotide Probes," hereby incorporated by reference herein.

T_m can be measured using HPA in the following manner. Oligonucleotides are labeled with an acridinium

ester. Oligonucleotide:target hybrids are formed in a lithium succinate buffer (0.1 M lithium succinate buffer (pH 5.0), 2 mM EDTA, 2 mM EGTA, 10% (w/v) lithium lauryl sulfate) using an excess amount of nucleic acid target.

5 Aliquots of the solution containing the nucleic acid hybrids are then diluted in the lithium succinate buffer solution. The aliquots are incubated for five minutes at various temperatures starting below that of the anticipated T_m and increasing in 2-5°C increments. This

10 solution is then diluted with a mild alkaline borate buffer (0.15 M sodium tetraborate, (pH 7.6), 5% (v/v) TRITON™ X-100) and incubated at a lower temperature for ten minutes. Under these conditions the acridinium esters attached to single-stranded oligonucleotides are

15 hydrolyzed, while acridinium esters attached to hybridized oligonucleotides are relatively protected from hydrolysis. Thus, the amount of acridinium ester molecules remaining after hydrolysis treatment is proportional to the number of hybrid molecules present in the sample. The remaining

20 acridinium esters can be measured by monitoring the chemiluminescence produced by addition of hydrogen peroxide and alkali to the solution. Chemiluminescence can be measured in a luminometer (e.g., the Gen-Probe LEADER I® or LEADER 50®). The resulting data is plotted as

25 percent of maximum signal (usually from the lowest temperature) versus temperature. In this assay, the T_m is determined to be the temperature at which 50% of the maximum signal remains. In addition to the method above, T_m may be determined by isotopic methods well known to

30 those skilled in the art (see e.g., Hogan et al., supra).

The optimal oligonucleotide size of a targeted oligonucleotide should take into account different factors including different mechanisms of action and cellular uptake. Targeted oligonucleotides are preferably 18 to

35 100 nucleotides, more preferably, 18 to 50 nucleotides in length, and most preferably, 20-35 nucleotides in length.

Oligonucleotides containing longer nucleic acid sequences of complementarity to a target sequence offer several advantages compared to shorter oligonucleotides, including increased target specificity and increased stability of the oligonucleotide:target duplex. The increased stability of the oligonucleotide:target duplex may facilitate the oligonucleotide nucleic acid inhibitory effect in different manners. For example, if the primary effect is translation arrest, the increased stability of the duplex could increase translation arrest by preventing a ribosome from displacing the oligonucleotide.

Another example of a possible mechanism involves degradation of the RNA strand of an DNA:RNA HIV duplex with an enzyme having RNase H activity. In this instance, the increased stability of the duplex increases the likelihood that the duplex is acted on by the enzyme. To be degraded by RNase H activity, the anti-HIV oligonucleotide in the DNA:RNA HIV duplex preferably contains three or more contiguous phosphodiester or phosphorothioate linkages.

Possible disadvantages of oligonucleotides having a longer length include a decrease in oligonucleotide uptake and a possible increase in host toxic effect. The degree of these effects are, at least in part, determined by the size of the oligonucleotide and types of oligonucleotide linkages. Possible host toxic effects may be more pronounced, for example, using oligonucleotides containing phosphorothioate linkages.

Without being bound to any theory, the use of two targeted oligonucleotides is expected to exert their effect by inhibiting a particular RNA nucleic acid more than either targeted oligonucleotide alone. Further inhibition may arise due to hybridization to different forms of RNA or to more efficient inhibition of a particular form of RNA.

Non-Targeted and Targeted Oligonucleotide Combination

A non-targeted and targeted oligonucleotide combination is preferably used to inhibit a retrovirus. Non-targeted oligonucleotides are longer length oligonucleotides joined by phosphorothioate linkages. Preferably, the non-targeted oligonucleotide is 20 to 100 nucleotides in length, more preferably 25 to 50 nucleotides in length.

Without being bound to any theory, non-targeted oligonucleotides are thought to function in a non-sequence specific manner primarily by inhibiting reverse transcriptase. However, as noted above, longer chain phosphorothioate oligonucleotide may also increase uptake of a targeted oligonucleotide into a cell. Thus, while non-targeted phosphorothioate oligonucleotide are particularly useful for inhibiting retroviruses such as HIV, there is no intention to limit the use of non-targeted phosphorothioate oligonucleotides to inhibit a retrovirus.

Sense and aptamer oligonucleotides

Sense oligonucleotides are oligonucleotides having the same nucleic acid sequence as a nucleic acid sequence present on a targeted nucleic acid. Sense oligonucleotides are directed to a particular protein target. Aptamers (Ellington and Szostak, Nature 346:812 (1990)) are oligonucleotides selected for their ability to bind to specific protein target. Aptamers directed to a specific target can bind to the specific target. Aptamers may be made up of RNA and/or DNA and may be single or double-stranded.

Without being bound to any particular theory, sense oligonucleotides are thought to inhibit nucleic acid activity by inhibiting proteins involved in nucleic acid activity. Proteins involved in nucleic acid activity
5 include proteins such as ribosomal binding proteins and tRNA synthetase which help bring about the production of a protein from nucleic acid (e.g., proteins involved in transcription and/or translation).

Similarly, aptamers are thought to function by
10 binding specifically to the active site of enzymes or other macromolecules and inhibiting the normal biological activity of their targets.

Combinations of sense or aptamer oligonucleotides and targeted oligonucleotides may display synergy when
15 targeted to, and directed to, the same or different nucleic acid or protein target. For example, the combination of a targeted oligonucleotide targeted to a nucleic acid, and a sense oligonucleotide directed to a protein involved in nucleic acid activity, may inhibit the
20 targeted nucleic acid more than the sum of the inhibition of the targeted and sense oligonucleotide acting alone.

A targeted/sense or aptamer oligonucleotide combination can also be used to inhibit production of more than one protein involved in a biosynthetic or regulatory
25 pathway. Such a combination should be more effective in inhibiting the multiplication of a foreign organism than either compound used alone.

Thus, a targeted oligonucleotide/sense or aptamer oligonucleotide combination, like other oligonucleotide
30 combinations described herein (e.g., a two targeted oligonucleotide combination, a targeted oligonucleotide/enhancer combination or a targeted oligonucleotide/non-targeted oligonucleotide combination) may be more effective than either oligonucleotide acting
35 alone because either oligonucleotide acting alone may not completely block a biosynthetic or regulatory pathway, or completely inhibit a particular nucleic acid. However,

inhibiting the production of several proteins of a pathway, or a single nucleic acid in multiple ways, may completely, or almost completely, block the pathway or inhibit the particular nucleic acid.

5 Methods for selecting sense or aptamer oligonucleotides directed against a specific protein are known in the art. Examples of these methods include those methods described by Mills et al. Proc. Natl. Acad. Sci. USA 58:224 (1967), Green et al. Nature 347:406 (1990), and
10 Tuerk and Gold Science 249:505 (1990), which show the use of *in vitro* evolution techniques to select RNA molecules with desired properties. Ellington and Szostak Nature 346:818 (1990), and Bock et al. Nature 355:564 (1992) describe the selection of DNA oligonucleotides. The field
15 has recently been reviewed by Burke and Berzal-Herranz FASEB J 7:106 (1993). These references are hereby incorporated by reference herein.

Therapeutic activity

One use of the present invention is to increase
20 the therapeutic efficacy of targeted oligonucleotides. The therapeutic use of targeted oligonucleotide/subtargeted oligonucleotide, two targeted oligonucleotides, or a targeted oligonucleotide/non-targeted oligonucleotide, combination generally involves
25 inhibiting target nucleic acid activity, more than essential cellular nucleic acid activity. Essential cellular nucleic acid activity is nucleic acid activity needed for cell growth and functioning.

Targeted oligonucleotides or subtargeted
30 oligonucleotides which cannot adequately discriminate between target nucleic acid and non-target essential cellular nucleic acid are less preferred because of possible cell toxicity. Generally, unwanted toxicity can be reduced or eliminated by decreasing the concentration
35 of a drug. However, the lower concentration may be therapeutically ineffective. The described oligonucleotide compositions acting synergistically

inhibit a target nucleic acid at a lower oligonucleotide concentration. As a result, oligonucleotides that might be too toxic at effective doses may be used effectively at a lower non-toxic concentration. Thus, the combination of
5 the two oligonucleotides may allow discrimination between target and non-target nucleic acid, even when the individual oligonucleotides do not adequately discriminate at their effective concentrations.

Before testing *in vivo*, the ability of a targeted
10 oligonucleotide or subtargeted oligonucleotide to hybridize to a particular target site can be assessed. For example, the hybridization behavior of an antisense oligonucleotide can be measured in essentially physiological conditions. Similarly, the ability of a
15 ribozyme to hybridize and cleave its target nucleic acid sequence can be assayed in essentially physiological conditions.

Exact duplication of *in vivo* conditions is extremely difficult because the intracellular environment
20 is very complicated and not completely known. Furthermore, there are many nucleic acid binding proteins present in cells, which may effect accessibility and activity of target sequences.

In vivo hybridization activity can be modeled in
25 *vitro* (e.g., using standard solutions that approximate condition found in cells or extracellular fluids). Hybridization can be determined using an *in vitro* quantitative assay, according to Kohne U.S. Patent No. 4,851,330, or Maniatis et al. Molecular Cloning, (New
30 York, Cold Spring Harbor Press, 1982) or an oligonucleotide screening assay.

The oligonucleotide screening assay can be used to test the ability of oligonucleotides to hybridize to a target sequence under different conditions. The assay is
35 preferably carried out by contacting a nucleic acid target sequence with an oligonucleotide, adding RNase H, then measuring the amount of remaining target sequences using

an acridinium ester-labeled probe under stringent hybridization conditions. Nelson et al., "Detection Of Acridinium Esters By Chemiluminescence" in: Nonisotopic DNA Probe Techniques, (Kricka ed., Academic Press, 1992) pp. 275-311, hereby incorporated by reference herein, describe using AE labeled probes to assay for target nucleotide sequences.

The acridinium ester attached to a single-stranded oligonucleotide is susceptible to hydrolysis and rendered non-chemiluminescent under mild alkaline conditions. However, the acridinium ester present in hybridized nucleic acid (e.g., probe:mRNA) is relatively resistant to hydrolysis. Hybridization of probe:mRNA can be measured by the chemiluminescence produced from the acridinium ester upon the addition of hydrogen peroxide followed by alkali. Chemiluminescence can be measured in a luminometer (e.g., LEADER I™, LEADER 50™, LEADER 250™ and LEADER 450™ these luminometers are available from Gen-Probe Incorporated).

An oligonucleotide screening assay can be carried out as follows:

- 1) Hybridize oligonucleotides to their target nucleic acids in a solution, such as an aqueous physiological buffer. An example of a target nucleic acid is purified HIV mRNA. Hybridization can be carried out using 0.9 pmol of target mRNA, 0.1 pmol acridinium ester-labeled probe, in 100 μ L of a physiological buffer, at 37°C for 2 hours. The reactions are divided to make duplicates at 1 x final buffer concentration for optimal RNase H enzyme activity.
- 2) *E. coli* RNase H (Life Technologies, Gaithersburg, MD, 0.4 U/reaction) is added to one of the two duplicate reactions. The other duplicate reaction lacks RNase H and serves as the (-) RNase H control. The

reactions are incubated at 37°C for 1 hour, stopped by denaturing at 95°C for 5 minutes, and placed directly on ice.

- 5 3) Aliquots of the reaction mixtures are hybridized with the appropriate phosphodiester acridinium ester-probe. Appropriate acridinium ester-labeled probes can hybridize to the same nucleic acid sequence as the test oligonucleotide and contain an acridinium ester in the complementary region. The acridinium ester-labeled probe is hybridized at 60°C for 1 hour. Control hybridization reactions are performed using acridinium ester-probes expected to hybridize to a region other than the target nucleic acid sequence.
- 10
- 15
- 20 4) Aliquots are diluted in hybridization buffer (0.1 M lithium succinate buffer (pH 5.0), 2 mM EDTA, 2 mM EGTA, 10% (w/v) lithium lauryl sulfate). Fifty microliter replicates are hydrolyzed in 12 x 75 mm luminometer tubes with 300 μ L of 0.15 M sodium tetraborate (pH 7.6), 5% (v/v) Triton X-100 at 60°C until non-hybridized labeled probes are fully hydrolyzed (usually 6-8 minutes). Chemiluminescence is brought about using a single injection of 1.5 N NaOH, 0.1% H₂O₂ and measured in a luminometer.
- 25

As would be appreciated by one skilled in the art, variations of this procedure can be performed. For example, the assay can be carried out using different amounts of reagents and incubation times.

30

If the compositions described herein inhibit essential cellular nucleic acid activity, they may still be able to be used as therapeutics by being targeted to certain types of cells. For example, liposomes containing recognition molecules targeted to HIV-infected cells may

35

be used to deliver the therapeutic composition to only HIV-infected cells.

Synthesis Of Oligonucleotides

Oligonucleotides containing phosphodiester linkages as well as modified linkages can be synthesized by procedures known in the art. For example, Caruthers *et al.*, in: Methods In Enzymology, vol. 154, p. 287 (1987), describe using standard phosphoramidite solid-phase chemistry to join nucleotides by phosphodiester linkages; Bhatt, U.S. Patent No. 5,252,723 describes a procedure for synthesizing oligonucleotides containing phosphorothioate linkages; and Klem *et al.*, entitled "Improved process for the synthesis of oligomers" PCT WO92/07864, describe the synthesis of oligonucleotides having different linkages including methylphosphonate linkages.

Administration of Oligonucleotides

The present invention may be used as a human therapeutic for treating different afflictions including viral infections, bacterial infections, parasitic infections, and cell proliferation diseases. The described oligonucleotides can be used to treat patients using different formulations and routes of administration. Suitable routes of administration include intramuscular, aerosol, oral (tablet, liquid suspension, or pill form), topical, systemic, ocular, intravenous, intraperitoneal and intrathecal.

Pharmacological considerations such as toxicology, pharmacokinetics, absorption, distribution, metabolism and excretion should also be taken into account. These considerations relate to the ability of the oligonucleotide to reach its target site, inhibit the propagation of a foreign organism or host nucleic acid, and possibly cause side effects adversely affecting patients. Different pharmacological considerations can be evaluated using techniques known in the art.

The described oligonucleotides may be introduced as naked oligonucleotides, through expression vectors

(encoding the oligonucleotide), by use of iontophoresis, or as physiologically acceptable formulations. Suitable formulations include the use of a liposome, a controlled release vehicle, ion paired molecules, covalently attached
5 adducts, and other pharmacologically suitable methods of delivery.

Different types of delivery strategies are useful in the present invention, including oligonucleotide modifications, particle carrier drug delivery vehicles,
10 and retroviral expression vectors. Oligonucleotides joined by phosphodiester linkages are slowly taken up by cells. To enhance cellular uptake, the oligonucleotide may be modified at the phosphodiester linkages to reduce its charge. For example, the individual nucleotides may
15 be joined by methylphosphonate linkages or phosphorothioate linkages.

Modification of oligonucleotides to reduce charge is just one approach to enhance the cellular uptake. Modifications can also be designed to reduce
20 susceptibility to nuclease degradation (e.g., use of methylphosphonate or phosphorothioate linkages).

Drug delivery vehicles can be chosen for both systemic and topical administration. These vehicles can be designed to serve as a slow release reservoir or to
25 deliver their contents directly to the target cell. An advantage of using some direct delivery drug vehicles is that multiple molecules are delivered per uptake. Such vehicles have been shown to increase the circulation half-life of drugs that would otherwise be rapidly cleared from
30 the blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes, hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres.

The use of liposomes as drug delivery vehicles
35 offers several advantages. Liposomes increase intracellular stability, increase uptake efficiency and improve biological activity. Liposomes are hollow

spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. They have an internal aqueous space for entrapping water soluble compounds and range in size from
5 0.05 to several microns in diameter. Several studies have shown that liposomes can deliver nucleic acids to cells and that the nucleic acids remain biologically active. For example, a liposome delivery vehicle originally designed as a research tool, Lipofectin, can deliver
10 intact nucleic acid molecules to cells.

Specific advantages of using liposomes include the following: they are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be readily attached to their
15 surface for targeting to tissues. Finally, cost-effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system.

20 The described oligonucleotides may be systemically administered. Systemic absorption refers to the entry of drugs into the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include:
25 intravenous, subcutaneous, intraperitoneal, and intranasal. Each of these administration routes delivers the oligonucleotide to accessible diseased cells. Following subcutaneous administration, the therapeutic agent drains into local lymph nodes and proceeds through
30 the lymphatic network into the circulation. The rate of entry into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier localizes the oligonucleotide at the lymph node. The oligonucleotide can be modified to
35 diffuse into the cell, or the liposome can directly participate in the delivery of either the unmodified or modified oligonucleotide into the cell.

A liposome formulation which can associate oligonucleotides with the surface of lymphocytes and macrophages is also useful. This will provide enhanced delivery to HIV-infected cells by taking advantage of the
5 specificity of macrophage and lymphocyte immune recognition of infected cells.

Intraperitoneal administration also leads to entry into the circulation with the molecular weight or size of the oligonucleotide-delivery vehicle complex
10 controlling the rate of entry.

The chosen method of delivery will result in entry into the afflicted cells, and molecules should have some nuclease resistance for optimal dosing. Most preferred delivery methods include liposomes (10-400 nm),
15 hydrogels, controlled-release polymers, and other pharmaceutically applicable vehicles, and microinjection or electroporation (for ex vivo treatments).

The exact dosage and number of doses will depend upon the data from clinical trials. Several factors such
20 as the delivery vehicle, disease indication, the route of administration, and the linkage joining the oligonucleotide will affect the dosage. The expected dosage is between 0.001-200 mg/kg of body weight/day. The duration of treatment will extend at least through the
25 course of the disease symptoms.

Establishment of therapeutic levels of oligonucleotides within the cell is dependent upon the rates of uptake and efflux degradation. Decreasing the degree of degradation prolongs the intracellular half-life
30 of the oligonucleotide. Thus, chemically-modified oligonucleotides, e.g., with modification of the phosphate backbone, may require different dosaging.

AIDS therapeutic

Oligonucleotides can be targeted to different
35 HIV activities. Targeting is generally based on an HIV nucleic acid sequence. However, some targeting can also be achieved by using oligonucleotides containing

phosphorothioate linkages. Phosphorothioate linkages increase oligonucleotide stability, facilitate oligonucleotide uptake, and enable the oligonucleotide to inhibit HIV propagation by a mechanism which appears to be largely sequence independent. Thus, phosphorothioate linked oligonucleotides can inhibit HIV by targeting, based on their nucleic acid sequence, a specific HIV target site, and inhibit other HIV target sites by a mechanism not dependent on a specific nucleic acid sequence.

Oligonucleotides having phosphorothioate linkages inhibit viral reverse transcriptase, and may also inhibit gp120 binding to CD4 receptor and phosphorylating activity of PKC. The viral reverse transcriptase inhibitory effect increases as the size of the phosphorothioate oligonucleotide increases, and appears to be applicable for other viral reverse transcriptases. Oligonucleotides having phosphorothioate linkages are described by Cohen et al., U.S. Patent 5,264,423, and Kinchington et al., Antiviral Research, 17:53-62, 1992.

An example of an HIV target site is the HIV nucleic acid sequence denoted by SEQ. ID. NO. 5 ("target site 1"). Target site 1 is present in exons coding for tat, rev, env, and nef. The presence of the same target site in more than one gene is due to overlapping exons in the HIV genome. As a result of the overlapping exons several genes in the HIV genome contain many of the same nucleic acid sequences (Schwartz et al., Journal of Virology 64:2519 (1990)).

Target site 1 is a conserved region of the HIV genome expected to have a secondary structure. A secondary structure arises from the formation of intramolecular hydrogen bonds between complementary nucleotides. The weaker the secondary structure, the less likely the formation of intramolecular hydrogen bonds.

A conserved region is a nucleic acid region present in at least three different strains of HIV which

can hybridize to a particular oligonucleotide *in vivo* such that the nucleic acid activity of in each strain of HIV will be inhibited. Conserved regions can be identified using standard techniques known in the art (e.g.,
5 comparing the primary nucleic acid sequence of different HIV strains then testing for hybridization to a particular oligonucleotide). Preferably, conserved regions have no more than a 10 % percent difference in nucleotide sequence between two different strains of HIV.

10 Target site 1 has several nucleotide regions that would be expected to be involved in formation of secondary structure. In particular, target site 1 has regions that contain three contiguous C residues and one region containing four contiguous G residues. These
15 regions may be involved in the formation of a secondary structure.

Secondary structure is associated with target function. Accordingly, hybridization to regions of secondary structure can be a particularly effective means
20 for inhibiting nucleic acid activity. However, a problem with targeting secondary structure is forming an oligonucleotide:target duplex. The intramolecular hydrogen bonding creating the secondary structure is expected to inhibit the formation of an
25 oligonucleotide:target duplex.

Anti-HIV oligonucleotides can effectively function as therapeutic agents by inhibiting viral propagation without gravely affecting the patient. In use as a therapeutic, it is possible there will be some
30 adverse side effects. An overall therapeutic effect can be obtained by providing an overall benefit to the patient, such as increased life expectancy and increased responsiveness of the immune system. Adverse effects can be reduced using standard medical techniques and include
35 considerations such as dosage regime. For example, anti-HIV oligonucleotides having a low therapeutic index can be

used at lower concentrations over a continuous time period.

Anti-HIV oligonucleotides which cannot adequately discriminate between HIV and essential cellular nucleic acid, and inhibits HIV nucleic acid to the same extent that it inhibits essential cellular functions, are less preferred. Such oligonucleotides may be able to act as a therapeutic by being delivered to only HIV infected cells (e.g., using liposomes containing recognition molecules targeted to HIV infected cells).

Oligonucleotide toxicity can be evaluated before therapeutic administration using models such as cellular assays and test animals. Cellular assays can be used to measure the cytotoxic effect of an agent and its ability to inhibit HIV propagation. (For example, see Weislow et al., J Natl Cancer Inst 81:577-586, 1989.) Preferably, test animals are used to measure the toxicity of anti-HIV oligonucleotides.

Examples are provided to illustrate different aspects and embodiments of the present invention and is not intended in any way to limit the disclosed invention.
Example 1: Inhibition of HIV

The ability of a subtargeted oligonucleotide and an antisense oligonucleotide to synergistically inhibit HIV multiplication is shown below using HT4-6C cells infected with HIV. An antisense oligonucleotide was targeted to target site 1. The nucleic acid sequence of the antisense oligonucleotide is given by SEQ. ID. NO. 1. The subtargeted oligonucleotide, whose nucleic acid sequence is given by SEQ. ID. NO. 3 is a truncated version of SEQ. ID. NO. 1. The nucleotide groups of both the subtargeted oligonucleotide and the antisense oligonucleotide were joined by phosphorothioate groups and contained deoxyribose moieties.

HT4-6C cells were detached using trypsin-EDTA for 8-10 minutes at 37°C. The cells were then centrifuged at 100 rpm for 10 minutes. Cells were resuspended in 5-10

ml of maintenance medium (Dulbecco's Modified Eagle Medium containing 10% fetal calf serum, 500 units/mg penicillin, 500 units/mg streptomycin, and 2 mM glutamine). Cells were maintained by splitting once weekly into two 75 cm² T-
 5 flasks at a concentration of 3×10^4 cells/ml in a total volume of 200 ml maintenance medium.

On day 0, the cells were plated into the wells of a 24 well tissue culture plate (Falcon #3047) at a concentration of 2.4×10^4 cells per well. The cells were
 10 incubated overnight at 37°C. The next day the medium was aspirated, and 200 μ l of HIV virus at a multiplicity of infection (MOI) of 0.03 was added to each well. The plates were then incubated for 2 hours at 37°C. After the 2 hour incubation, the cells were treated with 800 μ l of
 15 drug at a concentration of 1.25x (in Dulbecco's Modified Eagle Medium), of the final concentration. Antisense oligonucleotide/subtargeted oligonucleotide combinations were used at different ratios. Table 1 shows antisense oligonucleotide:subtargeted oligonucleotide combinations
 20 at a ratio of 1:1 and 10:1.

The treated cells were incubated for 3 days at 37°C. On day 4 the plate was fixed with 1 ml/well of 100% methanol for 15 minutes. The wells were then aspirated and 0.5 ml of 0.3% crystal violet stain was added to the
 25 wells. After 5 minutes the wells were washed with water and dried. Plaques were counted using a microscope.

TABLE 1

COMPOSITION CONCENTRATIONS (nM)		# PLAQUES	% REDUCTION
SEQ. ID.	20,000	4	98
30 NO. 1	5,000	9	94
	1,250	28	83
	310	54	67
	78	120	26

37

COMPOSITION CONCENTRATIONS (nM)		# PLAQUES	% REDUCTION
SEQ. ID. NO.	20,000	16	90
3	5,000	24	85
	1250	63	61
5	310	103	36
	78	157	2
COMPOSITION CONCENTRATIONS (nM)		# PLAQUES	% REDUCTION
SEQ. ID. NOS.	20	1	99
1 and 3	5	9	94
10 10:1 ratio	1.25	11	93
	0.31	20	88
	0.078	85	47
COMPOSITION CONCENTRATIONS (nM)		# PLAQUES	% REDUCTION
SEQ. ID. NOS.	40	4	97
15 1 and 3	10	3	98
1:1 ratio	2.5	16	90
	0.62	24	85
	0.16	91	43
COMPOSITION CONCENTRATIONS (nM)		# PLAQUES	% REDUCTION
20 AZT	1,000	5	97
	316	18	89
	100	35	80
	31.6	46	71
	10	86	47

25

The listed concentrations refer to the combined amount of oligonucleotides. The number of plaques is an average number of the plaques per well. The percent reduction is the number of the plaques observed per well containing drug divided by the number of plaques well containing no drug (161 plaques/well).

30

As seen in Table 1, the combination of the subtargeted oligonucleotide and the antisense oligo-

nucleotide was about 1,000x more potent than either oligonucleotide acting alone. The antisense oligonucleotide alone appeared to inhibit HIV replication better than subtargeted oligonucleotide alone, but not as well as AZT. HIV inhibition with the antisense oligonucleotide/subtargeted oligonucleotide combination was greater than with AZT. The ability of the antisense/subtargeted oligonucleotide combination to inhibit HIV replication appeared to be affected by the targeted oligonucleotide to subtargeted oligonucleotide ratio. A ratio of 10 to 1 was more effective than a ratio of 1 to 1.

Example 2: In Vivo Toxicology

In vivo toxicology can be carried out in test animals by examining the ability of an oligonucleotide combination to cause gross morphological changes in organ or blood tissue. For example, a toxicology study can be carried out as follows:

- 1) Oligonucleotides are injected into a test mouse by the intra-tail vein. A control mouse receives a control solution lacking oligonucleotides.
- 2) The effect of the tested oligonucleotides on organs, tissues, and blood parameters are measured at different time points (e.g., t=0 and at 24 hours).

Blood parameters indicative of a toxic effect include changes in the number of platelets, red blood cells, white blood cells, and hemoglobin. Examples of important organs and tissues to monitor to gauge toxic effect are spleen, liver, kidney and thymus.

Example 3: HIV Protection Assay

This example describes an anti-HIV production assay to determine the presence of HIV and screen for oligonucleotide combinations effective in protecting against HIV. The same types of assays can be used for

different foreign organisms, such as viruses and bacterium, to assay for effective oligonucleotides.

An HIV protection assay can be carried using anti-HIV oligonucleotides and standard techniques measuring cell growth. Techniques measuring cell growth include the use of dyes such as 2,3-bis[2-methoxy-4-nitro-5-sulphenyl]-2-H-tetrazolium-5-carboxanilide (XTT, Sigma Chemical Co.) to measure the cells metabolic state, the use of radioactive or modified nucleotide precursors such as BUdR (bromodeoxyuradine) to measure nucleic acid replication, and the use of oligonucleotides complementary to host nucleic acids to measure production of host nucleic acids.

Assays involving host complementary oligonucleotides to measure cell growth can be carried out using an oligonucleotide containing a detectable label such as fluorescent, chemiluminescent, enzyme or radioactive label. Oligonucleotides can be designed to hybridize to host nucleic acid sequence regions such as those present in DNA, mRNA or rRNA. Examples of such nucleotide sequence regions are known in the art and can be obtained by standard techniques. The preferred source of host target nucleic acids is rRNA. A nucleic acid having a nucleotide sequence characteristic of rRNA is generally present in a cell in much greater abundance than a nucleic acid sequence present in mRNA.

HIV infects cells containing a CD4 antigen (CD4⁺). The major target sites are T-helper lymphocytes and cells of the monocyte/macrophage lineage. The HIV-protection assay can be preformed on such cells from persons suspected of being infected with HIV. The assay can be preformed directly on such cells, or can be preformed using lysates obtained from CD4⁺ cells. The lysate can be used to infect cells more susceptible to HIV cytopathic effects than the isolated cells.

An HIV protection assay can be carried out as follows:

- 1) Isolate CD4⁺ cells from a person. Preferred cells are T-lymphocytes.
- 2) Incubate cells under conditions compatible with cell growth in the presence (treated cells) and absence (control cells) of an anti-HIV oligonucleotide combination. Examples of conditions compatible with cell growth are described by S. Gartner and M. Popovic, 1990, Virus Isolation and Production, pp. 53-70 in Techniques in HIV Research, ed. by A. Alaldocini and B.D. Walker. Stockton Press. N.Y.
- 3) Measure the growth of the treated and control cells at one or more time point after exposure of cells to the oligonucleotide combination.

Normal growth of control cells indicates the absence of a viral infection such as an HIV infection. Normal growth can be determined by comparing the growth of the control cells to the same type of cells which are known to be healthy.

Less than normal growth of control cells indicates the presence of some cellular disorder, such as HIV. The ability of an anti-HIV oligonucleotide to protect against HIV cytotoxicity in treated cells indicates the disorder is due to HIV and that the tested anti-HIV oligonucleotide combination can be used to treat the patient.

Due to the variability in nucleic acid sequences of different strains of HIV, the inability of an anti-HIV oligonucleotide combination to inhibit HIV cell toxicity in this assay may at times fail to indicate correctly the presence of HIV. Thus, an HIV protection assay should be used along with other assays known in the art to detect the presence of HIV. The assay is preferably used to make an initial determination regarding the suitability of a specific oligonucleotide to treat a patient infected with

41

HIV. Patients determined to be infected with HIV by other assays, but not by the HIV protection assay, should be retested using the HIV protection assay in conjunction with a different oligonucleotide combination.

5 Other embodiments are with the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Nanibhushan Dattagupta, C.
5 Nagaraja Sridhar, and Daniel
L. Kacian

(ii) TITLE OF INVENTION: ENHANCEMENT OF
10 OLIGONUCLEOTIDE INHIBITION
OF PROTEIN PRODUCTION, CELL
PROLIFERATION, AND/OR
MULTIPLICATION OF INFECTIOUS
DISEASE PATHOGENS

(iii) NUMBER OF SEQUENCES: 6
15

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Lyon & Lyon
20 (B) STREET: 611 West Sixth Street
(C) CITY: Los Angeles
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 90017

25 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb storage
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: IBM P.C. DOS (Version 6.0)
30 (D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
35 (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

40 Prior applications total,
including application
described below: 1

(A) APPLICATION NUMBER: 08/093,800
45 (B) FILING DATE: July 19, 1993

(viii) ATTORNEY/AGENT INFORMATION:

5 (A) NAME: Heber, Sheldon
(B) REGISTRATION NUMBER: P-38,179
(C) REFERENCE/DOCKET NUMBER: 208075

(ix) TELECOMMUNICATION INFORMATION:

10 (A) TELEPHONE: (213) 489-1600
(B) TELEFAX: (213) 945-0440
(C) TELEX: 67-3510

(2) INFORMATION FOR SEQ ID NO: 1 :

15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
20 (D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION : SEQ ID NO:1

CTTCGGGCCT GTCGGGTCCC CTCGGG 26

(3) INFORMATION FOR SEQ ID NO: 2 :

25

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26
(B) TYPE: nucleic acid
30 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION : SEQ ID NO:2:

CUUCGGGCCU GUCGGGUCCC CUCGGG 26

35

(4) INFORMATION FOR SEQ ID NO: 3 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14
40 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

44

(ii) SEQUENCE DESCRIPTION : SEQ ID NO: 3 :

GCCTGTCGGG TCCC

14

(5) INFORMATION FOR SEQ ID NO: 4 :

(i) SEQUENCE CHARACTERISTICS:

5

(A) LENGTH: 14
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10

(ii) SEQUENCE DESCRIPTION : SEQ ID NO: 4 :

GCCUGUCGGG UCCC

14

(6) INFORMATION FOR SEQ ID NO: 5 :

(i) SEQUENCE CHARACTERISTICS:

15

(A) LENGTH: 26
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20

(ii) SEQUENCE DESCRIPTION : SEQ ID NO: 5 :

CCCGAGGGGA CCCGACAGGC CCGAAG

26

(7) INFORMATION FOR SEQ ID NO: 6 :

(i) SEQUENCE CHARACTERISTICS:

25

(A) LENGTH: 26
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30

(ii) SEQUENCE DESCRIPTION : SEQ ID NO: 6 :

GCTGGTGATC CTTTCCATCC CTGTGG

26

CLAIMS

1. A method for inhibiting protein production from a nucleic acid comprising the step of providing to
5 said nucleic acid a composition comprising a purified targeted oligonucleotide targeted to a nucleic acid sequence region present on said nucleic acid and a purified subtargeted oligonucleotide, wherein said subtargeted oligonucleotide comprises a truncated version
10 of said targeted oligonucleotide.

2. The method of claim 1, further comprising a second purified subtargeted oligonucleotide, wherein said second subtargeted oligonucleotide comprises a different truncated version of said targeted
15 oligonucleotide than said subtargeted oligonucleotide.

3. The method of claim 1, wherein said composition inhibits protein production at least 10 fold more than said targeted oligonucleotide and said subtargeted oligonucleotide acting alone.

20 4. The method of claim 3, wherein said subtargeted oligonucleotide or said targeted oligonucleotide comprise at least one linkage selected from the group consisting of phosphorothioate linkage, phosphodiester linkage, and methylphosphonate linkage.

25 5. The method of claim 4, wherein said subtargeted oligonucleotide and said targeted oligonucleotide each comprise at least one phosphorothioate linkage.

6. A method for inhibiting protein production from a nucleic acid comprising the step of contacting said nucleic acid with two purified oligonucleotides targeted to different nucleic acid sequence regions present on said
5 nucleic acid coding for said protein wherein said nucleic acid sequence regions are more than 3 nucleotides apart.

7. A method for inhibiting the multiplication of a first organism present within a cell or body fluid of a second organism comprising the step of contacting
10 nucleic acid of said first organism with two purified oligonucleotides targeted to different nucleic acid sequence regions of said foreign organism wherein said nucleic acid sequence regions are more than 3 nucleotides apart.

15 8. The method of claim 7, wherein said first organism is selected from the group consisting of bacteria, fungi, parasites, and viruses.

9. The method of claim 8, wherein said different nucleic acid sequence regions both comprise a
20 region of the HIV genome.

10. The method of claim 9, wherein each of said purified oligonucleotides comprise at least one linkage selected from the group consisting of phosphorothioate linkage, phosphodiester linkage, and methylphosphonate
25 linkage.

11. The method of claim 10, wherein each of said purified oligonucleotide comprise at least one phosphorothioate linkage.

12. A method for inhibiting the multiplication
30 of a first organism present within a cell or body fluid of a second organism comprising the step of contacting

nucleic acid of said first organism with a purified targeted oligonucleotide targeted to said first organism nucleic acid and a purified subtargeted oligonucleotide, wherein said subtargeted oligonucleotide comprises a truncated version of said targeted oligonucleotide.

13. The method of claim 12, further comprising a second purified subtargeted oligonucleotide, wherein said second purified subtargeted oligonucleotide comprises a different truncated version of said targeted oligonucleotide than said subtargeted oligonucleotide.

14. The method of claim 12, wherein said first organism is selected from the group consisting of fungi, bacteria, parasites, and viruses.

15. The method of claim 14, wherein said nucleic acid comprises a region of the HIV genome.

16. The method of claim 15, wherein the ratio of said targeted oligonucleotide to said subtargeted oligonucleotide is about 10 to 1.

17. The method of claim 15, wherein said subtargeted oligonucleotide or said targeted oligonucleotide comprise at least one linkage selected from the group consisting of phosphorothioate linkage, phosphodiester linkage, and methylphosphonate linkage.

18. The method of claim 17, wherein said subtargeted oligonucleotide and said targeted oligonucleotide each comprise at least one phosphorothioate linkage.

19. A method for inhibiting multiplication of an organism comprising the step of contacting said

organism with a purified targeted oligonucleotide and a non-targeted phosphorothioate oligonucleotide, wherein said targeted oligonucleotide is targeted to a nucleic acid sequence region of said organism.

5 20. The method of claim 19, wherein said organism is a retrovirus.

 21. The method of claim 20, wherein said retrovirus is HIV.

 22. The method of claim 21, wherein said
10 targeted oligonucleotide comprises at least one linkage selected from the group consisting of methylphosphonate linkage, phosphodiester linkage, and phosphorothioate linkage.

 23. The method of claim 22, wherein the
15 predominant linkage of said targeted oligonucleotide is phosphorothioate linkage.

 24. A composition for inhibiting protein
production from a nucleic acid comprising a purified
targeted oligonucleotide targeted to a nucleic acid
20 sequence region present on said nucleic acid and a purified subtargeted oligonucleotide, wherein said subtargeted oligonucleotide comprises a truncated version of said targeted oligonucleotide.

 25. The composition of claim 24, further
25 comprising a second purified subtargeted oligonucleotide, wherein said second purified subtargeted oligonucleotide comprises a different truncated version of said targeted oligonucleotide than said subtargeted oligonucleotide.

26. The composition of claim 24, wherein the ratio of said targeted oligonucleotide to said subtargeted oligonucleotide is about 10 to 1.

27. The composition of claim 24, wherein said
5 subtargeted oligonucleotide or said targeted oligonucleotide comprise at least one linkage selected from the group consisting of phosphorothioate linkage, phosphodiester linkage, and methylphosphonate linkage.

28. The composition of claim 27, wherein said
10 subtargeted oligonucleotide and said targeted oligonucleotide each comprise at least one phosphorothioate linkage.

29. A composition for inhibiting protein
15 production from a nucleic acid comprising two purified oligonucleotides targeted to different nucleic acid sequence regions present on said nucleic acid, wherein said nucleic acid sequence regions are more than 3 nucleotides apart.

20 30. The composition of claim 29, further comprising a third targeted oligonucleotide, wherein said third targeted oligonucleotide is targeted to a different nucleic acid sequence region present on said nucleic acid than either of said two purified oligonucleotides.

25 31. A composition for inhibiting the multiplication of an organism comprising two purified oligonucleotides targeted to different nucleic acid sequence regions of said organism wherein said nucleic acid sequence regions are more than 3 nucleotides apart.

32. The composition of claim 31, wherein said organism is selected from the group consisting of fungi, bacteria, parasites, and viruses.

33. The composition of claim 32, wherein said
5 different nucleic acid sequence regions both comprise a region of the HIV genome.

34. The composition of claim 32, wherein each of said two purified oligonucleotides comprise at least one linkage selected from the group consisting of
10 phosphorothioate linkage, methylphosphonate linkage, and phosphodiester linkage.

35. The composition of claim 34, wherein each of said purified oligonucleotides comprise at least one phosphorothioate linkage.

15 36. A composition for inhibiting the multiplication of an organism comprising a purified targeted oligonucleotide targeted to an organism nucleic acid and a purified subtargeted oligonucleotide, wherein said subtargeted oligonucleotide comprises a truncated
20 version of said targeted oligonucleotide.

37. The composition of claim 36, further comprising a second purified subtargeted oligonucleotide, wherein said second purified subtargeted oligonucleotide comprises a different truncated version of said targeted
25 oligonucleotide than said purified subtargeted oligonucleotide.

38. The composition of claim 36, wherein said organism is selected from the group consisting of fungi, bacteria, parasites, and viruses.

39. The composition of claim 38, wherein said nucleic acid comprises a region of the HIV genome.

40. The composition of claim 39, wherein said subtargeted oligonucleotide or said targeted
5 oligonucleotide comprise at least one linkage selected from the group consisting of phosphorothioate linkage, phosphodiester linkage, and methylphosphonate linkage.

41. The composition of claim 40, wherein said subtargeted oligonucleotide and said
10 oligonucleotide each comprise at least one phosphorothioate linkage.

42. A composition for inhibiting protein production from a nucleic acid comprising a targeted oligonucleotide targeted to said nucleic acid and a sense
15 or aptamer oligonucleotide directed to a protein or other enzyme.

43. A composition for inhibiting the multiplication of an organism comprising a targeted oligonucleotide and a sense oligonucleotide or aptamer,
20 wherein said targeted oligonucleotide is targeted to a nucleic acid, and said sense oligonucleotide or aptamer is directed to an enzyme or other protein.

44. A composition for inhibiting the multiplication of an organism comprising a purified
25 targeted oligonucleotide and a non-targeted phosphorothioate oligonucleotide, wherein said targeted oligonucleotide is targeted to nucleic acid of said organism.

45. The composition of claim 44, further
30 comprising a purified subtargeted oligonucleotide, wherein

said subtargeted oligonucleotide comprises a truncated version of said targeted oligonucleotide.

46. The composition of claim 45, wherein said organism is a retrovirus.

5 47. The composition of claim 46, wherein said retrovirus is HIV.

 48. The composition of claim 47, wherein said targeted oligonucleotide comprises at least one linkage selected from the group consisting of phosphorothioate
10 linkage, phosphodiester linkage and methylphosphonate linkage.

 49. The composition of claim 48, wherein said targeted oligonucleotide comprises at least one phosphorothioate linkage.

15 50. A composition for treating a cell proliferative disorder comprising two purified targeted oligonucleotides targeted to two different nucleic acid sequence regions present in nucleic acid coding for a regulatory element responsible for said cell proliferative
20 disorder wherein said nucleic acid sequence regions are more than 3 nucleotides apart.

 51. A composition for treating a cell proliferative disorder comprising a purified targeted oligonucleotide and a purified subtargeted
25 oligonucleotide, wherein said purified targeted oligonucleotide is targeted to nucleic acid coding for a regulatory element responsible for said cell proliferative disorder and said subtargeted oligonucleotide comprises a truncated version of said targeted oligonucleotide.

52. A composition for inhibiting unwanted cell proliferation comprising a purified targeted oligonucleotide and a non-targeted phosphorothioate oligonucleotide, wherein said purified targeted
5 oligonucleotide is targeted to a nucleic acid coding for a regulatory element responsible for said cell proliferative disorder

53. A method of treating or preventing a cell proliferative disorder in humans comprising the step of
10 administering the composition of any of the claims 50-52 to a human.

54. A therapeutic composition for treating a person infected with HIV comprising a purified targeted oligonucleotide 9 to 100 nucleotides in length comprising
15 at least 9 contiguous nucleotides perfectly complementary to the HIV nucleic acid SEQ. ID. NO. 5: CCCGAGGGGA CCCGACAGGC CCGAAG, and a purified subtargeted oligonucleotide comprising a truncated version of said targeted oligonucleotide, wherein said therapeutic
20 composition is able to inhibit HIV multiplication.

55. The composition of claim 54, wherein said subtargeted oligonucleotide is 40 to 60 % the size of said targeted oligonucleotide.

25 56. The therapeutic composition of claim 55, wherein said targeted oligonucleotide has the nucleotide sequence of SEQ. ID. NO. 1: CTTGGGGCCT GTCGGGTCCC CTCGGG or the RNA equivalent thereof SEQ. ID. NO. 2: CUUCGGGCCU GUCGGGUCCC CUCGGG.

30 57. The therapeutic composition of claim 56, wherein said subtargeted oligonucleotide has the nucleotide sequence of SEQ. ID. NO. 3: GCCTGTCGGG TCCC or

54

the RNA equivalent thereof SEQ. ID. NO. 4: GCCUGUCGGG
UCCC.

58. The therapeutic composition of claim 55 wherein said subtargeted oligonucleotide or said targeted
5 oligonucleotide comprise at least one linkage selected from the group consisting of phosphorothioate linkage, phosphodiester linkage, and methylphosphonate linkage.

59. The therapeutic composition of claims 56 or 57, wherein said subtargeted oligonucleotide and said
10 targeted oligonucleotide each comprise at least one phosphorothioate linkage.

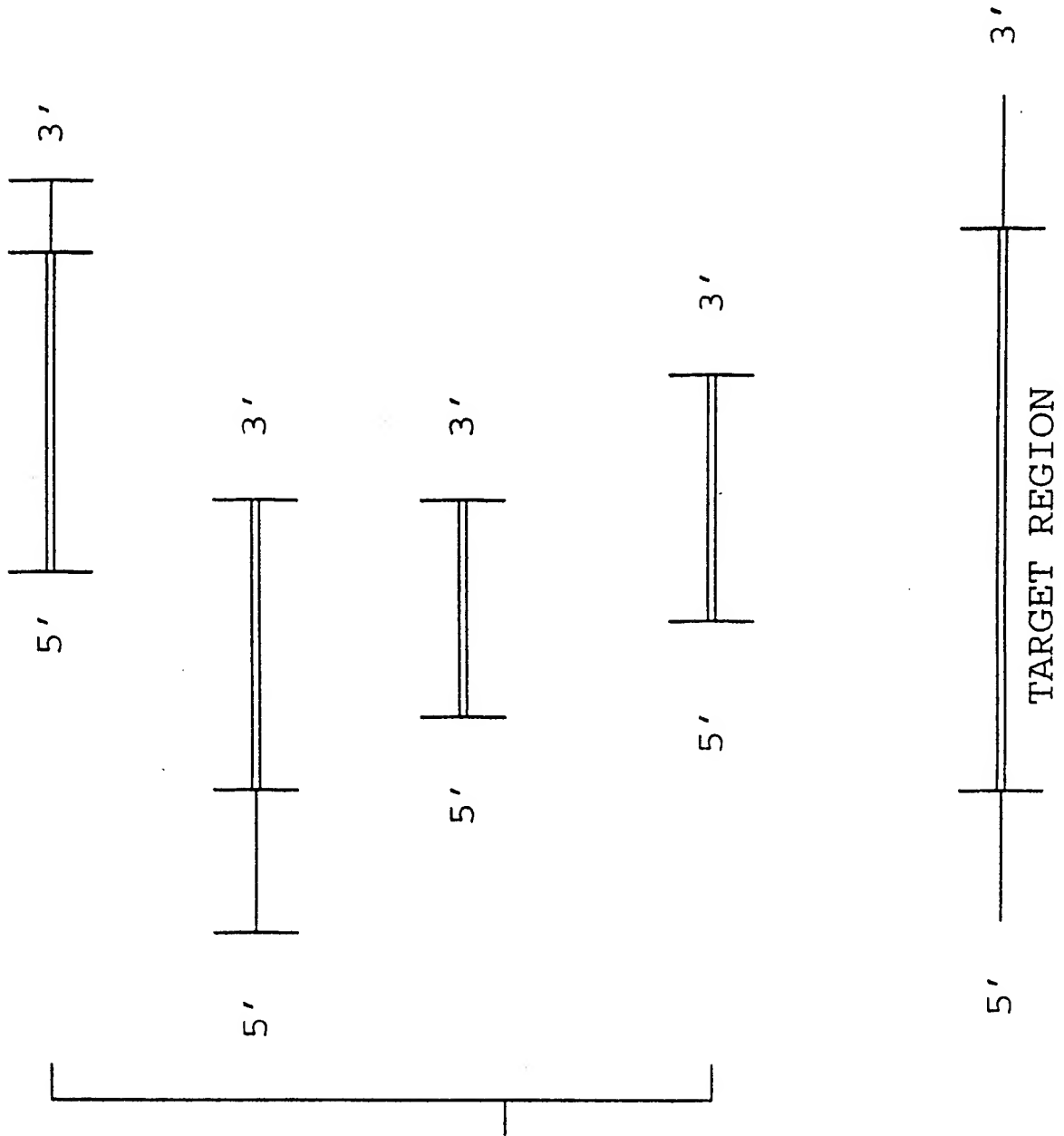
60. A method for treatment of a patient infected with HIV comprising the step of administering the therapeutic composition of claim 54 to said patient.

15 61. A therapeutic composition for treating a human affliction comprising two purified targeted oligonucleotides which act synergistically to effect at least 10 times the sum of the therapeutic effect of each oligonucleotide acting alone.

20 62. The therapeutic composition of claim 61, wherein said affliction is selected from the group consisting of unwanted cell proliferation, or infection by a organism.

1/2

FIG. 1.



SUBSET
TARGET REGIONS

SUBSTITUTE SHEET (RULE 26)

TARGET REGION

FIG. 2.

TARGET SITE-1 5' ——— CCCGAGGGGACCCGACAGGCCCGAAG ——— 3'

SUBTARGETED OLIGONUCLEOTIDE 3' CCCTGGGCTGTCCG 5' 2/2
(SEQ. ID. NO: 3)

TARGETED OLIGONUCLEOTIDE 3' GGGTCCCCCTGGGCTGTCCGGGCTTC 5'
(SEQ. ID. NO. 1)